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THE INTERACTION OF ADRIAMYCIN WITH SMALL UNILAMELLAR VESICLE LIPOSOMES

A FLUORESCENCE STUDY

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Summary

The interaction of the antineoplastic agent adriamycin with sonicated liposomes composed of phosphatidylcholine alone and with small amounts (1—6%) of cardiolipin has been studied by fluorescence techniques. Equilibrium binding data show that the presence of cardiolipin increases the amount of drug bound to liposomes when the bilayer is below its phase transition temperature and when the ionic strength is relatively low (0.01 M). At higher ionic strength (0.15 M) and above the T_m (i.e. conditions which are closer to the physiological state) the binding of the drug to the two liposome types is nearly the same. Thus the differences in the interactions of adriamycin with cardiolipin-containing membranes, as opposed to those composed of phosphatidylcholine alone, are not due simply to increased binding but rather to an altered membrane structure when this lipid is present. Quenching of adriamycin fluorescence by iodide shows that bound drug is partially, but not completely, buried in the liposomal membrane. Both in the presence and absence of cardiolipin the bulk of the adriamycin is more accessible to the quencher below the T_m than above it; that is, a solid membrane tends to exclude the drug from deep penetration. Above the T_m, the presence of cardiolipin alters the nature of the liposome-adriamycin interaction. Here the fluorescence quenching data suggest that the presence of small amounts of cardiolipin (3%) in a phosphatidylcholine matrix creates two types of binding environments for drug, one relatively

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Abbreviations: DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine.

exposed and the other more deeply buried in the membrane. The temperature dependence of the adriamycin fluorescence and the liposome light scattering reveal that cardiolipin alters the thermal properties of the bilayer as well as its interaction with adriamycin. At low ionic strength lateral phase separations may occur with both pure phosphatidylcholine and when 3% cardiolipin is present; under these conditions the bound adriamycin exists in two kinds of environment. It is notable that only adriamycin fluorescence reveals this phenomenon; the bulk property of liposome light scattering reports only on the overall membrane phase change. These data suggest that under certain conditions the drug binding sites in the membrane are decoupled from the bulk of the lipid bilayer.

Introduction

Adriamycin is an anthracycline antibiotic and an important agent in the treatment of human cancer. The drug intercalates with double-stranded nucleic acids and thereby inhibits their template-directed synthesis [1]. A variety of investigators have provided evidence of a role for the cell surface as an additional target for the action of adriamycin, and our laboratory has shown that the presence of the phospholipid cardiolipin may confer some specificity to the cytotoxic effects of the drug [2]. Regardless of which cellular entities are the ultimate receptor(s) for adriamycin, the surface membrane is the first barrier encountered and thus it is of interest to understand the mechanisms by which the drug interacts with membranes. In this paper we take advantage of the intrinsic fluorescence of the anthracycline moiety to probe some details of the interaction of adriamycin with phospholipid bilayers containing phosphatidylcholine and cardiolipin.

Materials and Methods

Dimyristoyl phosphatidylcholine (DMPC) and dipalmitoyl phosphatidylcholine (DPPC) were obtained from Sigma Chemical Co. Cardiolipin was obtained from Miles Laboratories. All lipids were stored in a dessicator at −20°C. The purity of the lipids was tested by thin-layer chromatography on silica gel using $CHCl_3/CH_3OH/CH_3COOH/H_2O$ (25 : 15 : 4 : 2). No impurities were detected in any samples used, either before or after sonication.

Adriamycin (NSC 123127) was obtained from the Division of Cancer Treatment of the National Cancer Institute.

Liposome solutions were made in a phosphate buffer at pH 7.4. The high ionic strength buffer contains per l: 8.0 g NaCl, 0.2 g KCl, 2.16 g $Na_2HPO_4 \cdot 7H_2O$ and 0.2 g KH_2PO_4. The low ionic strength buffer contains per l: 1.08 g $Na_2HPO_4 \cdot 7H_2O$ and 0.1 g KH_2PO_4. The pH was adjusted with HCl or NaOH. The cation concentrations (neglecting phosphate ionization) in the high and low ionic strength buffers are 0.15 M and 0.01 M, respectively.

Sonicated liposomes (small unilamellar vesicles) were prepared as described previously [3] using a Laboratory Supplies Company bath-type sonicator.

Dialysis experiments were performed by allowing two different lipid solu-

tions to compete for adriamycin binding in a common reservoir for 72 h. Bound and free concentrations were obtained by fluorescence intensity compared to a standard curve.

Melting experiments. Adriamycin was added to 0.7 mM liposome solutions to obtain a final concentration of 18 μM. Under these conditions at least 85% of the drug is bound to liposomes. Because the relative fluorescence emission of bound drug is at least twice that of free, greater than 95% of the fluorescence intensity in these experiments is due to bound drug. The solutions were equilibrated at 37°C for 1 h and then added to a quartz microcuvette in the fluorescence spectrophotometer. The cuvette temperature was monitored with a thermistor in direct contact with the cuvette. The solution was heated continuously at a rate of 0.3°C/min, beginning at 8°C. Adriamycin was excited at 470 nm with an excitation slit width of 0.5 mm, and fluorescence was monitored at 580 nm.

Temperature-dependent scattering changes were measured analogously to fluorescence, except that the emission and excitation monochromators were set to the same wavelength, generally 436 nm.

Fluorescence quenching. Adriamycin and $Na_2S_2O_3$ were added to a 7 mM liposome solution in high ionic strength buffer; the final concentration of drug was 4.6 μM and the final $Na_2S_2O_3$ concentration was 20 mM. The stock quenching solution contained 2.5 M KI in high ionic strength buffer with 20 mM $Na_2S_2O_3$. We found it necessary to use unusually high concentrations of $Na_2S_2O_3$ (concentrations of approx. 10^{-4} are used by other investigators in similar experiments, e.g. [4]) because liposomes-containing cardiolipin appear to catalyze the oxidation of I^-. The adriamycin solution was placed in a cuvette in the jacketed cuvette holder of the fluorometer which was thermostated at the desired temperature. The initial fluorescence was measured, and aliquots of the quenching solution were added. The data were used to generate a Stern-Volmer plot which was corrected for dilution. At low temperatures the Raleigh scattering from the liposomes became appreciable and the scattering signal from a blank (liposomes only) was subtracted from the total signal to obtain a corrected fluorescence reading.

It should be stated that the leakage rate of adriamycin out of liposomes is less than 0.01 h^{-1} [5]. Thus in the present work any redistribution of the drug to the inside of liposomes should not cause any measurable fluorescence changes over the time scale of 30—60 min necessary for a quenching or melting experiment.

Results

Binding of adriamycin to liposomes

In order to know the extent of binding of drug to liposomes in any equilibrium mixture we have measured the binding affinity using a previously described fluorescence technique [6]. This method takes advantage of the fact that liposome-bound adriamycin has a higher fluorescence intensity than free adriamycin, thus allowing construction of binding isotherms. The curved Scatchard plot in Fig. 1 indicates either binding site inhomogeneity or an anticooperative binding mechanism produced by a changing surface potential. The curves for

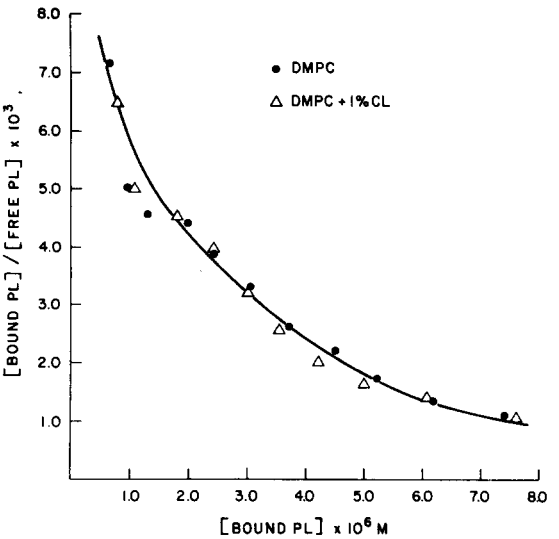

Fig. 1. Scatchard plots for the association of adriamycin with liposomes at 25° C in high ionic strength buffer. The data were obtained by titrating a fixed amount of adriamycin with varying amounts of sonicated liposomes. The equilibrium concentrations of free and bound species were obtained as described previously [6].

adriamycin binding to dimyristoyl phosphatidylcholine (DMPC) and DMPC with 1% cardiolipin are nearly coincident and yield an apparent limiting affinity constant of about $6 \cdot 10^3\ M^{-1}$ and three lipid molecules/adriamycin 'site'.

Equilibrium dialysis experiments could potentially provide a more accurate way to measure the adriamycin-liposome interaction. However, due both to problems of liposome aggregation and fusion, especially when acidic phospholipids are present [7], and to the fact that adriamycin tends to bind nonspecifically to dialysis or ultrafiltration membranes, we have not carried out quantitative dialysis experiments. Instead we have used dialysis competition between liposomes with and without cardiolipin to draw certain qualitative conclusions. Also, in order to assess the importance of electrostatic interactions in the binding of adriamycin competitive dialysis experiments were performed

TABLE I

EQUILIBRIUM DIALYSIS MEASUREMENTS OF THE MOLAR RATIO OF LIPOSOME BOUND TO FREE ADRIAMYCIN UNDER VARIOUS CONDITIONS

In every experiment the concentration and amounts of adriamycin and lipid are the same in order to allow direct comparisons. Each point is the average of three to six separate measurements with an estimated precision of ± 25%.

	Low ionic strength ($Na^+ + K^+ = 0.01$ M)			High ionic strength ($Na^+ + K^+ = 0.15$ M)		
	4°C	22°C	37°C	4°C	22°C	37°C
DMPC	1.8	1.8	2.0	2.5	2.1	5.1
DMPC + 6% cardiolipin	15	17	6.5	4.9	4.7	7.4

at low and high ionic strengths. The effect of membrane fluidity on binding was tested by performing dialysis at three different temperatures, above, below, and near the gel ⇌ liquid-crystal transition temperature. These results are summarized in Table I and discussed below.

Iodide quenches the fluorescence of adriamycin

Iodide ions (I^-) have been widely used as fluorescence quenching agents, especially in studies of tryptophan exposure in proteins [4,8]. Ionic quenchers are charged and heavily hydrated and consequently should only be able to quench surface residues or those in a relatively hydrophilic pocket. Because of this property we reasoned that I^- could be a useful probe of the accessibility of ligands bound to membranes. It has been shown that I^- penetrates lipid bilayers slowly if at all [9] and thus should be able to discriminate between molecules bound to the hydrophilic surface from those in the hydrophobic interior of a membrane. Classically, the relation used to describe dynamic quenching is the Stern-Volmer equation

$$\frac{F_0}{F} = 1 + K_{sv}[I^-]$$

where F_0 and F are the measured fluorescence intensities in the absence and presence of iodide at concentration $[I^-]$ and K_{sv} is the collisional quenching constant. The experimentally determinable quenching constant is equal to the product of the excited-state lifetime in the absence of quencher (τ_0) and the bimolecular rate constant for collision between the reactants (k)

$$K_{sv} = k\,\tau_0$$

If the fluorescence emission is heterogeneous, i.e. if more than one type of fluorophore is present, the Stern-Volmer plot may be non-linear and an exact description of the quenching process is complex. In general it is difficult to extract the exact fluorescence contributions and quenching constants for each fluorophore from such data, although it is possible to define certain limiting cases [10]. Four our purpose, when the quenching curves are non-linear we assume two classes of emitting species, where the slope at low $[I^-]$ is the weighted average of the individual quenching constants and is referred to as $K_{sv(1)}$. The limiting slope at high $[I^-]$ is a complex average of the fractional quenching efficiencies of the two forms and will be referred to as $K_{sv(2)}$; the y-intercept of this line gives a measure of the average fractional accessible fluorescence.

Fig. 2 shows a typical Stern-Volmer plot for the quenching of free adriamycin by I^-. The curve is linear as expected for a single type of emitting species. The quenching constant, K_{sv}, is 25 M^{-1} indicating that free adriamycin is very efficiently quenched by I^-. We assign this value of 25 M^{-1} as the standard of complete accessibility to quencher for comparison to later experiments.

Adriamycin bound to phosphatidylcholine liposomes is quenched less efficiently than free drug

Fig. 2 shows that adriamycin bound to dimyristoyl phosphatidylcholine liposomes, both below (11°C) and above (34°C) the phase transition tempera-

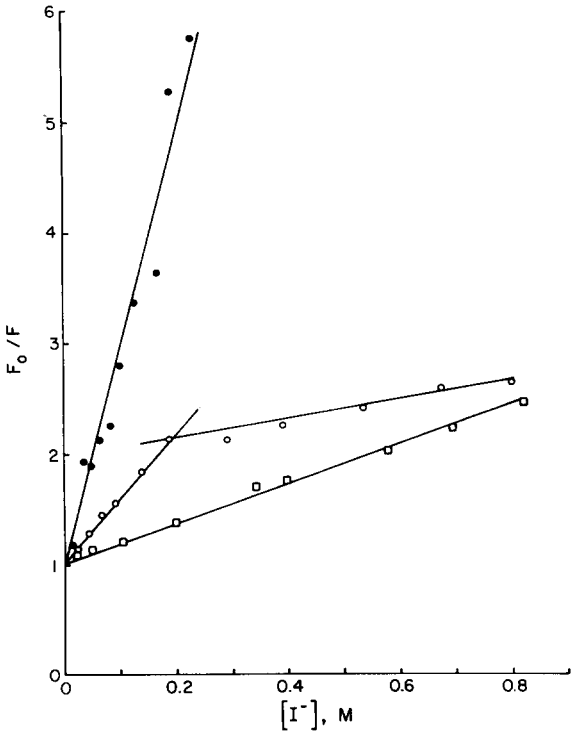

Fig. 2. Iodide (I^-) quenching of adriamycin fluorescence free in solution (•) and bound to DMPC liposomes at 34°C (□) and 11°C (○). F_0 is the limiting fluorescence intensity in the absence of quencher and F is the fluorescence in the presence of the indicated concentration of I^-. The data are presented in a Stern-Volmer plot as described under Materials and Methods. The phospholipid concentrations are 7.2 mM and adriamycin is 4 μM.

ture, is quenched by I^- but much less efficiently than free adriamycin in solution. In these experiments the drug is greater than 99% bound to insure that free drug does not contribute to the observed fluorescence. For fluid DMPC (above the T_m) the quenching curve is linear yielding a K_{sv} of 1.5 M^{-1}. Thus the bound drug is much less susceptible to quenching than free, but still maintains some degree of exposure to the solvent. For comparison, we have determined that anthracene, which is completely embedded in the lipid bilayer, is totally inaccessible to I^- quenching ($K_{sv} = 0$, data not shown).

The Stern-Volmer plot below the thermal phase transition for DMPC is biphasic (Fig. 2). To demonstrate that this is a result of the relative fluidity and not simply of the nature of DMPC or temperature, we also constructed a quenching curve for DPPC at 25°C. DPPC is below its T_m at this temperature and shows essentially identical behavior with respect to bound adriamycin quenching by I^- as DMPC at 11°C (Fig. 2). Accordingly, when the drug is attached to solid liposomes there are at least two different bound species.

The limiting quenching constant values for the two bound adriamycin species are about 6 and 1 M^{-1} for $K_{sv(1)}$ and $K_{sv(2)}$, respectively. The value of $K_{sv(1)}$ is not directly comparable to K_{sv} derived from the linear Stern-Volmer curves because the former is weighted somewhat by the second, less quenched,

type of adriamycin. However, $K_{sv(1)}$ is a lower limit to the true K_{sv} and thus this class of drug bound to solid liposomes is much more susceptible to quenching by I^- than that bound to fluid liposomes (compare $K_{sv(1)} = 6$ at 11°C with $K_{sv} = 1.5$ at 34°C for phosphatidylcholine). The second, less easily quenched, form of bound drug shows a $K_{sv(2)}$ value of 1 M^{-1} which represents an upper limit to the true K_{sv} for this class of adriamycin. Thus this species of bound adriamycin is almost, but not completely, unable to be quenched by I^-.

Because of the way these experiments were done, the ionic strength increases as the quencher (KI) is added to the solution. In principle one could maintain the ionic strength constant at the highest level to be reached in an experiment by using varying ratios of KI and a non-quenching salt like KCl. Because this would require a large investment of expensive lipids we have not performed the experiments this way. Instead, we conducted a control experiment with liposome-bound adriamycin and measured the fluorescence as we added increasing amounts of KCl alone. The concentration-corrected fluorescence emission remained constant over the entire range of salts from 0 to 0.8 M indicating that the fluorescence of liposome-bound adriamycin is not strongly dependent on ionic strength.

Liposomes containing a small amount (3%) of cardiolipin exhibit altered behavior with respect to quenching of bound adriamycin

The Stern-Volmer plots shown in Fig. 3 reveal that the presence of small amounts of cardiolipin in the phosphatidylcholine matrix causes the quenching of bound adriamycin to be biphasic at temperatures both above and below the thermal phase transition. Below the T_m, the cardiolipin membranes behave much like pure DMPC, exhibiting quenching constants $K_{sv(1)}$ and $K_{sv(2)}$ of, respectively, 5 and 1 M^{-1}. Above the T_m where DMPC itself shows linear quenching by I^-, Stern-Volmer plots for cardiolipin-containing DMPC liposomes are biphasic. Thus the presence of cardiolipin changes the behavior of adriamycin towards liposomes which are in the fluid state. Moreover, this effect is not simply a nonspecific result of the presence of acidic phospholipids in the bilayer because 3% phosphatidylserine in DMPC behaves like pure DMPC above the T_m (a single $K_{sv} = 2$ M^{-1}).

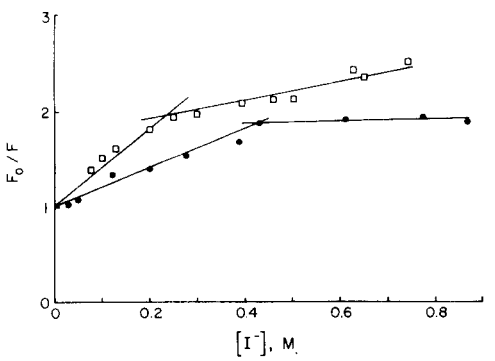

Fig. 3. I^- quenching of adriamycin bound to DMPC liposomes containing 3% cardiolipin at 34°C (•) and 11°C (□). The total lipid concentration is 7.2 mM and adriamycin is 4 μM.

Judging from the values of the various Stern-Volmer quench constants bound adriamycin is less easily quenched in fluid cardiolipin containing membranes than in solid ones ($K_{sv(1)}$ at 34°C and 11°C are 2 and 5 M^{-1}). This trend is seen also with pure DMPC, but in cardiolipin-containing liposomes there is a class of bound adriamycin which is completely inaccessible to I^- at 34°C ($K_{sv(2)} \rightarrow 0$). Accordingly, the presence of this phospholipid creates a membrane whose structure is sufficiently different from pure DMPC to create a unique type of binding site, probably more deeply buried in the bilayer, for adriamycin.

The temperature dependency of adriamycin fluorescence is an indicator of membrane events

We have used adriamycin as a fluorescent probe of liposomal structure at both high and low ionic strength. The rationale in these experiments is that the emission of the bound drug reports directly on its own microenvironment rather than on the bulk properties of the membrane measured by techniques like scattering or calorimetry. In this way we can then ask the questions, does the bound drug alter the overall organization of the membrane or is the effect contained in the vicinity of the binding site and to what extent are the adriamycin binding sites coupled to the bulk of the membrane?

Fig. 4 shows that for adriamycin bound to pure DMPC in high ionic strength buffer, the fluorescence increased rapidly up to about 19°C and then remains approximately constant except for a small but reproducible drop between 20 and 22°C. Our interpretation of this result is that below the DMPC phase transition (T_m = 23.9, Ref. 12) the mode of binding of adriamycin changes with temperature. This is consistent with the quenching data which show that as the temperature increases the adriamycin becomes less accessible to I^- quenching. Between 20 and 22°C the small change in the adriamycin fluorescence probably reflects the bulk thermal phase change in DMPC. Once the membrane is on the fluid side of the T_m the fluorescence of bound drug is

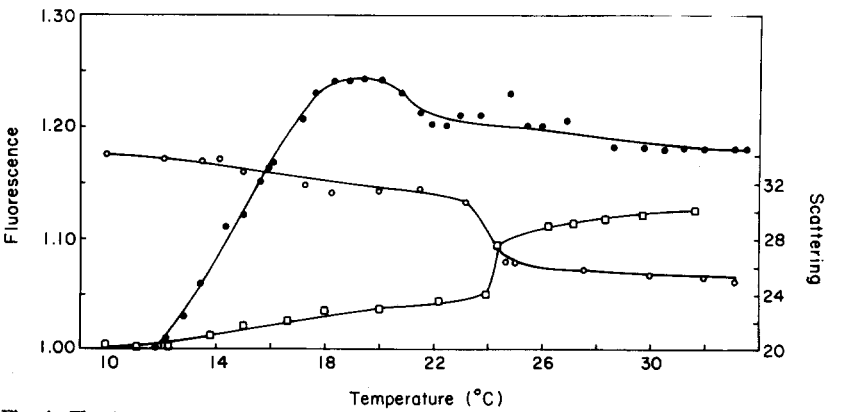

Fig. 4. The temperature dependence of adriamycin-bound liposomes in high ionic strength (0.15 M) phosphate buffer. Fluorescence measurement of (•) 0.7 mM DMPC and 18 μM adriamycin. Fluorescence (□) and scattering (○) measurements of 0.7 mM total DMPC (97%) + cardiolipin (3%) and 18 μM adriamycin.

essentially independent of temperature suggesting that its mode of binding is now constant in the fluid membrane.

Fig. 4 also shows 90° light scattering (which measures the bulk membrane properties) and adriamycin fluorescence (sensing the environment of the drug) as a function of temperature for DMPC containing 3% cardiolipin. The fluorescence data are quite unlike that obtained with pure DMPC showing only a slight monotonic increase with temperature and revealing a cooperative phase change at about 24°C. Thus the mode of binding of drug to cardiolipin-containing membranes does not change rapidly with temperature showing again that the interaction with this type of membrane is different than with pure DMPC. Also, consistent with our earlier work using turbidity to monitor phase transitions [2], adriamycin raises the T_m of DMPC membranes containing small amounts of cardiolipin. Comparing the 90° scattering curve with the fluorescence curve (Fig. 4), it is evident that the bulk membrane T_m (24°C) measured by scattering is very nearly the same as the T_m sensed by fluorescence at the adriamycin microenvironment. Thus any effect of a bound drug at high ionic strength is probably on the overall organization of the membrane and not localized to its immediate binding area.

At low ionic strength (phosphate buffer, no added salt), the temperature dependence of the optical properties yields somewhat different results. The light scattering and fluorescence temperature dependencies for adriamycin bound to pure DMPC liposomes are shown in Fig. 5. The scattering data reveal a bulk transition at about 25°C, a value somewhat higher than the T_m found at higher ionic strength. The fluorescence melting curve is clearly biphasic, with the second phase probably coinciding with the bulk, or overall, membrane gel-liquid crystal transition. The lower phase, centered at about 16°C, is not observed at high ionic strength and may reflect the fact that some segregation of lipid domains is occurring in this later case. It is notable that this phenomenon is not seen in the scattering curve so the effect is probably specific to the adriamycin microenvironment .

Fig. 6 shows scattering and fluorescence-detected transitions for 3% cardiolipin-containing DMPC liposomes in the presence of adriamycin in low ionic strength buffer. Light scattering reveals a T_m of about 23°C, slightly lower than

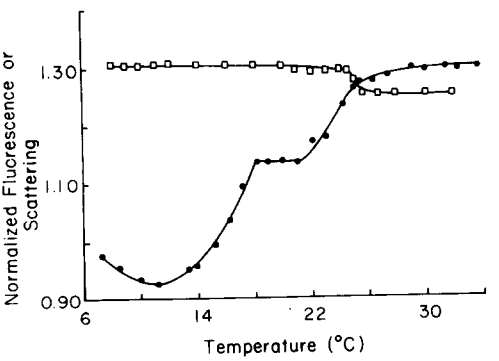

Fig. 5. The temperature dependence of scattered light (□) and adriamycin fluorescence (●) in low ionic strength (0.01 M) phosphate buffer. The conditions are 7 mM DMPC and 33 μM drug.

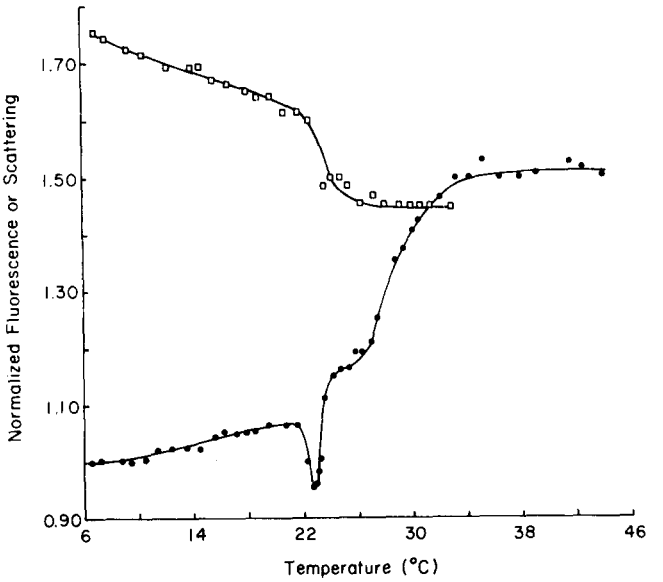

Fig. 6. The temperature dependence of scattered light (□) and adriamycin fluorescence (●) in low ionic strength (0.01 M) phosphate buffer. The conditions are 7 mM total phospholipid (97% DMPC + 3% cardiolipin) and 33 μM adriamycin.

in the absence of cardiolipin, as expected from previous work [2]. The fluorescence transition, on the other hand, shows a number of details not observed by scattering. In contrast to the result for pure DMPC the fluorescence intensity increases only very slowly with temperature up to 22°C. At about 22°C a dip in the curve appears much like that seen with pure DMPC at high ionic strength (Fig. 4), suggesting that some of the bound drug may be in a pure DMPC-rich neighborhood. Above 22°C a broad transition occurs centered at about 28°C, four degrees above the overall membrane phase change seen by scattering. It is clear from these results that the adriamycin molecules sense and report on different membrane events than are revealed by observing the overall properties of the membrane.

Discussion

Adriamycin, which is positively charged at physiological pH, has a moderate affinity for liposomal membranes which bear no net charge (phosphatidylcholine). The affinity is only slightly dependent on the concentration of metal ions in the solution, suggesting that forces other than ionic ones dominate the interaction. Also, a very weak temperature dependence of the binding process leads us to presume that the driving force for interaction may be largely entropic.

The presence of small amounts of the negatively charged phospholipid cardiolipin in a phosphatidylcholine matrix alters the interaction between adriamycin and liposomal membranes. At 1% cardiolipin the affinity of adriamycin for liposomes is not measurably different than with pure phosphatidylcholine membranes. Since the effect of the drug on the fluidity of these two

types of liposomes is in the opposite direction [2], we concluded that small amounts of cardiolipin had a unique long-range effect on the structure of the bilayer. In the present study we have utilized 6% cardiolipin in DMPC to further enhance the effect of this acidic phospholipid. In this case the binding of the drug is very dependent on the ionic strength of the solution, indicative of the importance of electrostatic contributions to the free energy of interaction. Likewise, 6% cardiolipin-containing liposomes bind 3—7 times more adriamycin than the neutral DMPC counterpart at low ionic strength, indicating that as the content of acidic phospholipids increases the affinity for adriamycin increases. However, the effect is diminished by increasing the salt concentration and thus the differential effect of adriamycin on liposomes at physiological ionic strength conditions (our high ionic strength buffer) is probably not due to a specific cardiolipin-adriamycin interaction on the membrane per se, but rather to an altered membrane structure in the presence of cardiolipin.

In low ionic strength buffer binding of adriamycin to DMPC/cardiolipin liposomes decreases sharply above the phase transition. This result can be rationalized by the fact that above the T_m a lateral expansion of the bilayer occurs [13]. This expansion results in a net increase in the volume/unit lipid and a concomitant decrease in the net charge density at the membrane surface. Since the coulombic attraction between ions is proportional to the charge density, one expects the amount of adriamycin bound to decrease above the T_m if electrostatic forces are dominant. This effect is much less important in the case of pure DMPC liposomes (Table I) where the net charge density on the surface is smaller and thus adriamycin binding by these liposomes in low ionic strength buffer does not change with the physical state of the membrane.

Duarte-Karim et al. [14] have shown that acidic phospholipids, including cardiolipin, will preferentially redistribute adriamycin into the lipophilic phase of a two phase solvent system. Also, Menozzi and Arcamone [15] demonstrated an affinity of the drug for sulfated mucopolysaccharides. The interaction in both instances is probably largely ionic since in the former case neutral phospholipids do not show the effect and in the latter study it could be shown that Na^+ disrupted the complex. Clearly the positively charged adriamycin will interact with materials bearing a negative charge; our results show in addition, however, that cardiolipin present in a membrane alters the interaction with adriamycin by changing the membrane structure, and not by simply acting as an anionic receptor. This effect is further emphasized by the recent finding by Serpentino [16] that about 70% of cardiolipin in a phosphatidylcholine liposome is asymmetrically disposed towards the inner monolayer, thus, effectively prohibiting a direct interaction with the surrounding medium.

The fluorescence quenching studies were undertaken to probe how the mode of adriamycin binding is different when cardiolipin is present in the liposomal bilayer. The rationale in these experiments is to see if I^- quenching can serve as a yardstick for the relative exposure of adriamycin bound to various membranes. It is clear that when bound to liposomes the drug is substantially less easily quenched than when free in solution. The chromophore is not completely buried in the bilayer however, as this situation would lead to a Stern-Volmer plot which is horizontal as seen with the hydrophobic probe anthracene. A reduced quenching constant could be due either to the bound adria-

mycin having a decreased fluorescence lifetime τ, or to a smaller value for the bimolecular quenching rate constant k_q. Only the latter quantity can be directly related to the degree of exposure in comparative quenching studies. We have not measured the lifetime of adriamycin but since binding of drug to liposome increases the fluorescence intensity we can infer that the bound adriamycin has a longer lifetime than free drug. By this argument we conclude that the decreased quenching constant, K_{sv}, seen for membrane-bound adriamycin must be due to a decreased bimolecular quenching rate constant and thus the determined values of K_{sv} are a useful measure of relative exposure. Consequently the drug is partially, but not completely, buried in the phospholipid bilayer. In a recent paper Goldman et al. [23] concluded from fluorescence polarization measurements that adriamycin was located at the hydrocarbon-water interface, consistent with the present quenching studies.

The quenching data also reveal that for both DMPC and DMPC containing cardiolipin, the bulk of the adriamycin is more accessible below (11°C) the thermal phase transition than above (34°C) it. Thus it appears that membranes in the gel phase exclude adriamycin from the more deeply embedded binding site which is preferred in the liquid-crystalline phase. However, both in the presence and absence of cardiolipin the quenching by I^- is heterogeneous and a fraction (about half) of the drug is fairly inaccessible to the quencher in the solid phase. The remainder of the bound drug in both instances, though, is effectively prevented from deep penetration into the interior of the liposomal bilayer.

The presence of cardiolipin in the bilayer changes the nature of the adriamycin liposome interaction (as sensed by I^- quenching) only when the membrane is above the T_m. Under these circumstances the quenching reagent reports a homogeneous population of DMPC-bound drug but at least two classes of DMPC/cardiolipin-bound drug. In the latter case the second class of adriamycin is almost completely inaccessible to I^- quenching. Thus the presence of cardiolipin allows a fraction of the drug to be essentially completely buried in the membrane. It is not likely that one of the classes of bound adriamycin is a specific complex with cardiolipin, both because the affinity for 3% cardiolipin liposomes is not substantially different than pure DMPC and because the ratio of cardiolipin to drug (approx. 50 : 1) would favor complete formation of such a complex, a situation not obtained. Thus the differential effect of cardiolipin is due to an altered membrane organization by this lipid rather than the creation of an anionic binding site. Moreover this is not simply a result of the fact that cardiolipin is an acidic phospholipid because DMPC membranes containing 3% phosphatidylserine (an acidic phospholipid) behave like pure DMPC showing homogeneous quenching above the T_m.

The measurement of liposome-bound adriamycin fluorescence as a function of temperature allows the drug to report on its own local environment. At high (0.15 M) salt the fluorescence of adriamycin bound to DMPC increases with temperature up to the T_m. This is probably not simply a reflection of an increase in binding since the binding constant favors greater than 95% bound under these conditions. Rather, our hypothesis is that as the temperature increases, an increasing fraction of the drug penetrates into the bilayer where it is inaccessible to quenching by proton transfer with the solvent [11], thus

leading to increased fluorescence *. Below the T_m, two forms of bound drug are present, with different degrees of accessibility and different intrinsic fluorescence quantum efficiencies. As the temperature is raised through the phase transition zone the equilibrium is shifted towards the less accessible, more deeply buried, more highly fluorescent binding mode.

The presence of 3% cardiolipin in the DMPC in the high ionic strength buffer alters this behavior. In this circumstance the fluorescence of bound adriamycin increases slowly with temperature over the entire range studied and also exhibits a discontinuity at the gel ⇌ liquid-crystalline point. This is consistent with the quenching results which showed that both above and below the T_m at least two classes of adriamycin are present, the constantly increasing fluorescence emission reflects the shift towards the less accessible form of the drug, but both classes are present at all temperatures studied. Thus the presence of small amounts of cardiolipin appears to allow adriamycin to be more deeply buried in the membrane.

In low ionic strength buffer both the nature of the membrane and its interaction with adriamycin is substantially different than at high ionic strength. In pure DMPC the adriamycin fluorescence versus temperature curve is now biphasic, the first aspect of which occurs several degrees below the bulk membrane T_m revealed by 90° light scattering. With cardiolipin present, on the other hand, the temperature curve is again represented by more than one phase and the fluorescence reports a membrane change which is several degrees above the overall membrane solid ⇌ fluid transition. If the adriamycin fluorescence curves in the presence and absence of cardiolipin are superimposed (not shown) it can be seen that the lower and upper transitions, respectively, lie at about 23°C and thus probably are a result of the overall membrane phase transition.

The explanation we favor for these results is that in low ionic strength buffer, both in the presence and absence of cardiolipin, the bound adriamycin senses more than one kind of membrane environment, hence the curves exhibit more than a single phase transition. That is, lateral phase separations may occur, with the adriamycin partitioning into and reporting on different local neighborhoods within the membrane. This occurs even when the membrane is composed of a homogeneous phospholipid (DMPC) and may be due specifically to the action of adriamycin since other fluorescent probes have not been reported to show this phenomenon (reviewed in Ref. 17). The origin of this probably stems from the increased repulsive forces between the charged lipid head groups at low ionic strength and a consequent decrease in the surface charge density. This allows the interaction of adriamycin with even a single component membrane to induce cluster formation and phase separation, similar to the results obtained with Ca^{2+} [22]. It is notable that the measurement of the bulk membrane property of light scattering does not reveal the details of the structural events detected by adriamycin. Apparently, even a simple, single component, membrane system may not always be a homogeneous sea of completely miscible components. Rather, segregation into specific

* We have shown that such a quenching mechanism is operative with both free and liposome-bound adriamycin by observing an increase in the fluorescence intensity when going from H_2O to ^2H_2O (data not shown).

neighborhoods can occur and the observation of such events depends on the method of detection.

In all the experiments reported here cardiolipin altered the nature of the adriamycin-liposome interaction. Other investigators too have discerned a unique role for this phospholipid. Both cytochrome oxidase [18] and cytochrome *c* [19] are postulated to form complexes with cardiolipin in the inner mitochondrial membrane and the latter enzyme can cause phase separations in cardiolipin-containing liposomes [20]. Also, Rand and Sengupta [21] have discussed the possibility that unusual hexagonal arrays of cardiolipin may be important in the regulation of membrane function. Previous work from this laboratory [2] has suggested that the presence of cardiolipin may provide some specificity in the cytotoxic action of adriamycin and we discussed there the evidence that in neoplasia the differentiated distribution of this phospholipid seen in normal cells is lost. It is clear from the present work that even relatively low amounts of this membrane component can have dramatic effects on a liposomal membrane structure, and on the interaction of such membranes with a therapeutic agent.

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